

# Damage assessment of parchment at the collagen fibril level using atomic force microscopy and mechanical testing at the macro level

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## INTRODUCTION

Parchment in the form of archival material bears the testimony of recorded cultural history. A recent paper focuses attention on the fact that large collections exist in national archival and religious institutions and their preservation presents an enormous unsolved conservation problem (Mühlen Axelsson et al. 2012). To assist in this process, it is important to understand the effect of environmental factors on parchment, in particular at the fibre and fibril level. Within the EC-funded IDAP project, Improved Damage Assessment of Parchment (FP5 IDAP VK4-CT-2001-00061), studies were made of the effect of fluctuations in relative humidity (RH) and temperature (T), and of inorganic pollutants on modern parchment. Preliminary results on some of these samples obtained from scanning electron microscopy (SEM), micro-thermal analysis (micro-TA) and some atomic force microscopy (AFM) have been previously reported (de Groot et al. 2005). SEM imaging identified ageing related to topography changes in the parchment surface of accelerated aged and historical samples. These changes were used to classify parchment into different categories where damage was accompanied with increase in formation of glass-like surfaces. Micro-TA, which uses a modified AFM probe and performs localised thermo-mechanical measurements on sample surfaces, showed transitions at both lower and higher temperatures. Studies on rat tail collagen identified the transitions at higher temperatures as those of gelatine and at lower temperature transitions as those of collagen (Bozec and Odlyha 2011). So the glass-like surface observed in SEM could be directly associated with surface gelatinisation. Examples of this were given in IDAP (Larsen 2007). In AFM, surface gelatinisation is seen as a loss in the periodic collagen structure (de Groot 2007).

In this paper, previously unreported results from the IDAP project are presented together with those from MEMORI, Measurement, Effect Assessment and Mitigation of Pollutant Impact on Movable Cultural Assets – Innovative Research for Market Transfer (FP7 no. 265132), which has considered the effects of internally generated volatile organic acids on organic-based cultural objects. Previous conservation treatment may also contribute to this internally degrading effect. The rationale for this study was based on the fact that levels of organic acids were found to be significantly higher within than outside enclosures and exceeded

**KEYWORDS:** damage assessment, preventive conservation, conservation treatment, parchment, collagen, atomic force microscopy

## ABSTRACT

The aim of this paper is to describe how atomic force microscopy (AFM) has been used to assess damage in parchment at the collagen fibril level on exposure to relative humidity (RH) fluctuations at a given temperature, and to pollutants. A method was developed for quantification of AFM images and data were found to correlate with shrinkage temperature of parchment fibres. The effects of collagen denaturation were also observed by micro-thermal analysis and at the macromolecular level by controlled-environment dynamic mechanical analysis. Internally generated volatile organic acids in storage enclosures are considered as the recent MEMORI project showed that their presence can lead to a lowering of pH, resulting in collagen denaturation and surface gelatinisation. The latter will have a different response to fluctuations in RH and temperature, and further damage will occur. In the current NANOFORART project, novel nanoparticle formulations have been developed and applied for adjustment of pH in parchment.

recommended levels (Grøntoft et al. 2011). However, these values have been taken from damage threshold levels for metals (e.g. lead) and one of the aims was to determine values for parchment as there is an increase in the use of enclosures for protecting objects. This is seen as an environmentally favourable solution which can contribute to climate change mitigation as it can replace energy-demanding climate conditioning using HVAC systems for cultural heritage building interiors. It is therefore important to encourage the use of enclosures by assuring optimal conditions for the objects they protect. The work in MEMORI has shown that acetic acid exposure changes fibre morphology and hydrothermal stability. Swelling and a lowering of pH was also observed (Dahlin 2013).

Within the NANOFORART project, Nanomaterials for the Conservation and Preservation of Movable and Immovable Artworks (FP7 no. 282816), one of the aims is to protect collagen-based artefacts with novel nanoparticle formulations. The basic idea is to adjust pH and prevent the collagen from being exposed to low or high pH values at which damage occurs (Bowes 1950). Previously some tests have been made on deacidification of leather bookbindings (Larsen et al. 2005). In NANOFORART the aim is to test the efficacy of nanoparticle preparations. This is the first time that such an approach has been used for parchment and consideration was given to the nature of nanoparticles and solvent type. Nanoparticles based on  $\text{Ca(OH)}_2$  and  $\text{CaCO}_3$  were used as calcium compounds were already present in the parchment from the liming process. Prior to the tests the particles were fully characterised (Melita 2013). Preliminary results on their application to modern parchment are reported.

To summarise, the following data are presented:

- 1 changes recorded for samples exposed to (a) fluctuating conditions in RH at a selected T and (b) externally generated inorganic pollutants ( $\text{NO}_2$ ,  $\text{SO}_2$ )
- 2 changes as observed in organic-acid exposed samples which simulate damage that could occur under improper storage conditions
- 3 preliminary observations on the effect of possible preventive or localised conservation treatment using novel nanoparticle formulations.

## MATERIALS AND METHODS

Modern calf parchment (Pergamena, US) dehaired in  $\text{Ca(OH)}_2$  with added NaHS to speed up the dehairing process and a historical parchment dated 1848 donated from the National Archives in Kew, UK were used.

### Sample ageing and preparation

Samples were aged (1) by heating at 80°C and 40% RH for 32 days and (2) by heating at 80°C with RH cycled between 40 and 80% RH every 2 days. Samples were also aged by exposure to inorganic pollutants ( $\text{NO}_2$ ,  $\text{SO}_2$ ) at concentrations of 50 ppm and at 50% RH (Larsen 2007). Furthermore, samples were exposed to acetic acid (400 mg/m<sup>3</sup> and 75% RH) (Odlyha et al. 2012). Thermal ageing was performed at 120°C for 48 and 96 hours and also by heating samples in a dynamic mechanical analyser at 3°C/min to 150°C and up to 230°C.

Bundles of fibrils were removed and moistened in water as performed by conservators in preparation for shrinkage temperature measurements. Fine tweezers were used to spread the softened bundles of fibrils over a glass coverslip. The fibres were physisorbed onto the glass coverslip and examined by optical microscopy and by AFM.

#### Treatment with alkaline nanoparticles

Nanoparticles  $\text{Ca}(\text{OH})_2$  in cyclohexane and  $\text{CaCO}_3$  in propan-2-ol were applied by brushing and by controlled addition using a Gilson pipette to the flesh side of unaged parchment samples. Some preliminary tests were also performed with calcium carbonate nanoparticles in aqueous-based solvents.

#### Atomic force microscopy and collagen structure

AFM is an imaging technique where a cantilever with a sharp tip ( $\sim 10$  nm in radius) at its end is raster-scanned on a surface, providing information to re-create a 3-D contour image. Imaging was performed using a Dimension 3100 (Bruker, USA) and a XE-100 of PSIA (Park Systems, Korea) in contact mode using cantilever C of a gold-coated microlever (Bruker) with scan rates of 1 or 2 Hz. The Nanosurf EasyScan 2 AFM in dynamic mode was also used. It was fitted with a cantilever (Nanosensors PPP-NCLR) with a spring constant of  $k_{\text{tip}} = 48$  N/m, resonance frequency of  $f_{\text{res}} = 170$  kHz and a tip radius of  $r_{\text{tip}} < 10$  nm.

#### Controlled environment dynamic mechanical analysis (DMA)

Controlled environment DMA provides information on the change in mechanical properties with RH. In the IDAP project, the rate of displacement change with controlled increase in RH provided a marker of damage (Odlyha et al. 2009). Prior to testing, samples were pre-dried and then mounted in the tensile clamp of the DMA (TRITEC 2000 B). The Triton RH controller unit was set at the start to 20% RH and 25°C. The RH was increased at 1%/min until 80% RH. Sample dimensions were typically 5 (or 10) (l)  $\times$  4.50 (w)  $\times$  0.2 (t) mm.

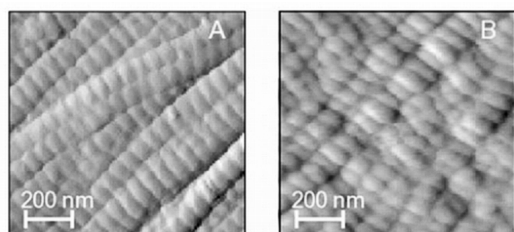
#### pH measurement

Samples (0.05–0.1 mg) were weighed and then placed in a vial. Distilled water (100  $\mu\text{l}$ ) was added using a Gilson pipette and the sample was left for 24 hours. pH was measured using the stainless steel ISFET pHW47-SS probe.

## EXPERIMENTAL RESULTS

#### AFM of accelerated aged samples

The application of AFM has brought greater understanding of the structure of collagen, the main component of parchment. According to the quarter staggered model theory, staggered arrays of collagen molecules form fibrils with regular axial periodic ‘D-banding’ pattern that is independent of fibril diameter. A recent paper gives the key to all mechanical properties of collagen with the hypothesis that (tendon) collagen fibrils are composed of subcomponents in a spiral disposition such as nanoscale ropes (Bozec et al. 2007).



**Figure 1**

Left (A) unexposed parchment shows intact banded fibril structure and right (B) aged (32 days at 80°C and 40% RH) shows less regular banding

AFM images of undisturbed collagen structure reveal a regular structure (Figure 1). On thermal denaturation, gelatine is formed. AFM images of gelatine show formation of a glassy layer that is not reminiscent of the collagen fibrillar topology (de Groot 2007). Studies on unaged parchment provided a starting point for understanding the appearance of collagen fibrils in parchment. Measurements were made of the D-banding and deviations were found in samples that had been damaged through exposure to elevated temperature and relative humidity. Regions of gelatinisation are also observed with absence of D-banding. In order to perform a fast Fourier transform (FFT) analysis of each image, an algorithm was developed to calculate the extent of surface coverage having intact banding periodicity and this was expressed as a peak area: the higher the peak area value, the greater the surface coverage with intact D-banding (de Groot 2007).

AFM images of unaged and aged samples at 80°C, 40% RH for 32 days are shown in Figure 1 (de Groot et al. 2005). Intact banded fibril structure is observed for the unaged sample, and for the aged sample the banding along the fibrils appears less regular. These images are representative for both samples. The number of fibre locations examined was 25 and 28 respectively (Table 1). Micro-TA of the aged sample (SC155) has been previously reported (de Groot et al. 2005). Some of the curves had a lower transition temperature and the others gave a higher transition temperature which is indicative of some surface gelatinisation. This complements observations made on over a thousand parchment folios, including volumes of the Domesday Book in the National Archives in Kew (UK) (Larsen et al. 2011).

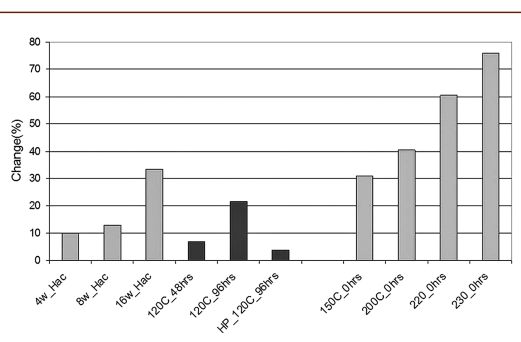
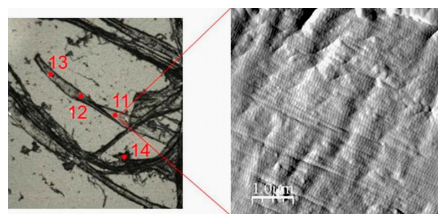
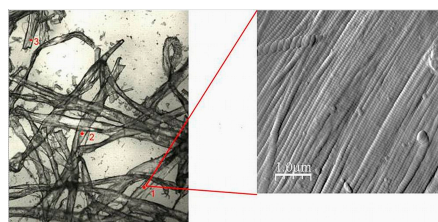
The FFT algorithm was used to calculate the extent of intact D-banding on the surfaces of the unaged and aged samples. Table 1 shows the results obtained. A decrease in peak area occurred with ageing, corresponding to a decrease in the extent of intact surface banding present in the image. Peak area values can be used as markers for collagen structural intactness; a decrease in the peak area was also found to be associated with a decrease in measured shrinkage temperature (Table 1).

**Table 1**

AFM peak area and shrinkage temperature decrease with loss of intact banded fibril structure

	Exposure (days)	Shrinkage temp. Ts (°C)	Peak area	No. of measurements
80 °C 40 to 80%RH every 2 days	0	55.9	9.1±1.6	10
	2	51.6	9.9±1.4	9
	4	49.6	5.4±1.8	13
	8	47.2	7.1±1.4	8
	16	43.9	5.8±1.2	13
	32	42.2	5.0±1.4	9
80 °C and 40%RH	0	58.7	9.6±1.1	25
	16	48.8	7.2±1.8	10
	32	46.0	5.8±1.7	28
50ppm SO <sub>2</sub> 50% RH	0	49.9	5.5±1.8	10
	28	45.0	0.7±0.3	10
	112	42.3	0.6±0.3	10
50ppm NO <sub>2</sub> 50%RH	112	45.0	1.7±0.7	10





**Figure 2**

Undamaged fibre (left) location 1 in unexposed sample provides AFM image (right)

**Figure 3**

Damaged fibre (left) location 11 exposed to acetic acid (2 weeks) provides AFM image (right)

**Figure 4**

Change (%) for samples exposed for 4–16 weeks to acetic acid (HAc), thermally aged and historical parchment (HP)

**Figure 5**

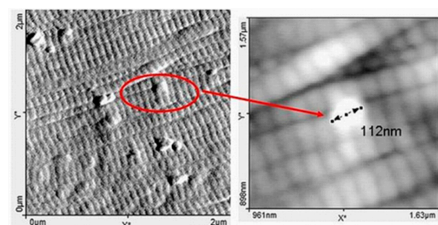
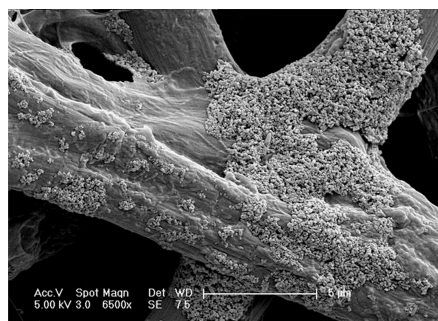
Optical microscopy thermally aged fibres (96 hours at 120°C)

Samples exposed to pollutant gases also displayed this change when analysed by the FFT algorithm. Values for NO<sub>2</sub> and SO<sub>2</sub> exposures showed a significant reduction in peak area relative to the reference (Table 1). The decrease was greater for SO<sub>2</sub> (87%) and NO<sub>2</sub> (69%) than for RH and T ageing alone (39%) for about a 4-week exposure. The banded fibril structure in the pollutant-damaged samples was almost completely absent in half of the locations examined and strongly degraded in the other half (10 sampling locations viewed in total). Samples for the ageing studies used three different hides and the unexposed samples show different starting values. Some may be more damaged due to the liming process.

### Organic pollutants

Exposure to acetic acid vapour at 75% RH showed pronounced changes in fibre morphology (Dahlin 2013). A detailed study was based on methods described elsewhere (Mühlen Axelsson et al. 2012). The photomicrographs and AFM images of the fibres from the unaged parchment before and after acetic acid exposure for two weeks are shown (Figures 2–3 respectively). Figure 3 shows the AFM image corresponding to a location in the fibre that demonstrates a deteriorated morphology with pearl structure which at the nanoscale level reveals fibril swelling, surface wrinkling, and localised areas of surface gelatinisation. The latter was confirmed by micro-TA where gelatine-like behaviour was observed (Odlyha et al. 2012). Application of the algorithm to AFM images showed that a 6-week exposure reduced the peak area with respect to its reference by 63%, and exposure for 8 weeks to 80% (Dahlin 2013). The reference was close in its location in the hide to the exposed sample and attention was paid to relating the location of the AFM image to the selected fibre on the photomicrograph. This means that the fibre assessment protocol developed at the School of Conservation, Copenhagen, and recently applied to historical parchment (Larsen et al. 2012) can now be extended to include AFM images.

There was also an effect on the mechanical properties. In the MEMORI project the damage parameter was calculated as the change (%) in displacement rate for each exposed sample and corresponding reference. Values are shown for the acetic-acid-exposed parchment, historical and thermally aged samples (Figure 4). The increase in change (%) is associated with increase in gelatinisation (Larsen 2007). The historical parchment shows lower damage than modern parchment as it starts in a more deteriorated state. The sample heated to 220°C has been reported elsewhere (Odlyha et al. 2009) and its AFM image showed loss of intact structure and significant decrease in displacement rate. For the sample aged for 96 hours at 120°C, fibre morphology changes are shown (Figure 5). There is a high amount of damaged fibres in the form of flat fibres as well as pearls on a string structure. For the acetic-acid-exposed samples, lowering of pH was observed which also varied with the period of degassing (Dahlin 2013). This causes the pH of the parchment to fall below the collagen isoelectric point, resulting in collagen denaturation and swelling. In addition to the acidic conditions, ageing was performed at 75% RH and this also provides suitable conditions for the collagen triple helix to unwind.



**Figure 6**

SEM of parchment treated with calcium carbonate nanoparticles

**Figure 7**

AFM of parchment treated with calcium carbonate nanoparticles

**Table 2**

pH values after addition of calcium carbonate nanoparticles to parchment (unaged and thermally aged)

Sample	pH
untreated	7.1
aged 48hrs at 120°C	5.9
aged 96hrs at 120°C	5.9
with CaCO <sub>3</sub>	7.3
aged 48hrs at 120°C	6.5
aged 96hrs at 120°C	6.6

## Effect of alkaline novel nanoparticles

An SEM image is shown of modern parchment after brushing with CaCO<sub>3</sub> nanoparticles in an aqueous dispersion and the nanoparticles can be seen on the fibres (Figure 6). The AFM image shows that the regular collagen structure is maintained and the particles can be seen and their size measured (Figure 7). This is similar to what is observed by transmission electron microscopy.

Additions of these nanoparticles to modern parchment showed changes in pH. The initial pH for modern parchment was about 7 and both Ca(OH)<sub>2</sub> nanoparticles (1 g/L) in cyclohexane and CaCO<sub>3</sub> (1.35 g/L) in propan-2-ol gave small increases in pH, but well below pH 8. Thermal ageing showed that in the case of parchment treated with CaCO<sub>3</sub> nanoparticles in propan-2-ol, the pH remains at the original value compared to aged untreated parchment where the pH is lowered (Table 2). The application of nanoparticles appears to prevent lowering of pH during ageing.

## CONCLUSIONS

This work has demonstrated that AFM and mechanical testing can provide valuable information on the level of damage incurred by parchment on exposure to environmental fluctuations and pollutants. In addition to the use of the algorithm and visual assessment of AFM images, it has been shown that the images can be linked to differences in fibre morphology. The MEMORI project has brought to awareness that improper storage conditions with elevated levels of volatile organic acids can lead to surface gelatinisation. The surface would then respond in a different manner to fluctuations in RH and temperature, which could lead to further damage.

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## MATERIALS LIST

Modern calf parchment

Pergamena

[www.pergamena.net](http://www.pergamena.net)

Dimension 3100, Cantilever C

Bruker

[www.bruker.com](http://www.bruker.com)

XE-100 of PSIA

Park Systems Corp.

Suwon, South Korea

[www.parkAFM.com](http://www.parkAFM.com)

Nanosurf® EasyScan 2 AFM

[www.nanosurf.com](http://www.nanosurf.com)

Nanosensors™ PPP-NCLR

Neuchâtel, Switzerland

[www.nanoworld.com](http://www.nanoworld.com)

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